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Exploring the contribution of fructophilic lactic acid bacteria to cocoa beans fermentation: Isolation, selection and evaluation

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ABSTRACT

Fructophilic lactic acid bacteria (FLAB) are a recently discovered group whose main characteristic is to prefer D-fructose over D-glucose. In this study, laboratory cocoa beans fermentation was analyzed by Illumina-based amplicon sequencing, indicating the presence of potential FLAB of the genera *Fructobacillus* and *Lactobacillus*. Eighty efficient fructose-fermenting isolates, obtained from fermenting cocoa pulp beans mass, were identified by 16S rRNA gene sequencing as *Pediococcus acidilactici* (n = 52), *Lactobacillus plantarum* (n = 10), *Pediococcus pentosaceus* (n = 10), *Bacillus subtilis* (n = 4), and *Leuconostoc pseudomesenteroides* (n = 4). The growth characteristics of all the 10 *L. plantarum* strains classified them as “facultatively” fructophilic bacteria, *i.e.,* they grew on glucose without an external electron acceptor but the growth on fructose was faster. Among them, *L. plantarum* LPBF 35 was characterized by producing a range of aroma-impacting compounds (acetaldehyde, ethyl acetate, nonanal, and octanoic acid), being introduced into a cocoa fermentation process. Although the process started with approximately equal amounts of glucose and fructose, a concomitant, but faster utilization of fructose, was observed in cocoa fermentation conducted with *L. plantarum* LPBF 35 (with no residual fructose observed) when compared to control fermentation using a glucophilic strain (8.77 mg/g residual fructose) and a spontaneous process (8.38 mg/g residual fructose). *L. plantarum* LPBF 35 also showed an ideal profile of organic acid metabolism (citric acid consumption and lactic acid production) associated with cocoa fermentation. These results proved new insights on cocoa microbial activity and brings new perspectives on the use of lactic acid bacteria as starter culture.

1. Introduction

Cocoa is the fruit of the *Theobroma cacao* L., a perennial tree native to the South American tropical region. Cocoa bean, the raw material for chocolate production, is composed of two cotyledons and an embryo enclosed by a seed coat, enveloped in a sweet and white mucilaginous pulp (Figueroa-Hernández et al., 2019). When cocoa is ripe, the pulp has a higher proportion of fructose and glucose; in addition to pectin and other polysaccharides, proteins, amino acids, minerals, vitamins and citric acid (Camu, De Winter, et al., 2008; De Vuyst & Weckx, 2016; Lima, Almeida, Rob Nout, & Zwietering, 2011). Once the cocoa beans are removed from the pod, the pulp is degraded by a natural fermentation process, which is mainly conducted by yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB). During fermentation, several metabolites are produced by microbial activity and diffused into seeds (Camu et al., 2007; Schwan & Wheals, 2004). This leads to a number of biochemical transformation within the seeds, reducing the bitter taste and astringency and, finally, killing the embryo to avoid its germination (Kadow, Bohlmann, Phillips, & Lieberei, 2013; Kadow, Niemenak, Rohn, & Lieberei, 2015). Thus, cocoa beans fermentation is considered to be the postharvest treatment stage that most influences the quality of chocolate.

The presence of LAB in the cocoa microbial consortium is associated with the anaerobic fermentation of simple sugars in the early stages of

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the process (0–48 h) and production of lactic acid, diacetyl, acetoin, 2,3-butane-diol, and other minor metabolites (Adler, Bolten, Dohnt, Hansen, & Wittmann, 2013; Camu et al., 2007; Camu, De Winter, et al., 2008; Castro-Alayo, Idrogo-Vásquez, Siche, & Cardenas-Toro, 2019; Figueroa-Hernández et al., 2019; Lefeber, Gobert, Vrancken, Camu, & De Vuyst, 2011; Lefeber, Janssens, Camu, & De Vuyst, 2010). The LAB species most frequently reported include the genera *Lactobacillus* and *Leuconostoc* (Camu et al., 2007; Figueroa-Hernández et al., 2019; Lagunes-Gálvez, Loiseau, Paredes, Barel, & Guiraud, 2007; Meersman et al., 2013; Ouattara et al., 2017; Serra et al., 2019; Visintin, Alessandria, Valente, Dolci, & Cocolin, 2016). Some attempts on designing LAB starter culture have been addressed for cocoa beans fermentation process (Kresnowati, Suryani, & Affifah, 2013; Lefeber et al., 2010; Lefeber, Janssens, Moens, Gobert, & De Vuyst, 2011). Based on knowledge of bacterial metabolism and physiology (lactic acid and volatile organic molecules production, citrate consumption, and heat and ethanol tolerance), two LAB species, *Lactobacillus fermentum* and *L. plantarum*, can be considered good candidates for starter culture (Adler et al., 2013; Camu et al., 2007; De Vuyst & Weckx, 2016; Figueroa-Hernández et al., 2019; Kresnowati et al., 2013; Lefeber et al., 2010; Lefeber, Gobert, et al., 2011). These species have been used in conjunction with yeasts (*Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Pichia kluyveri*, and *Kluyveromyces marxianus*) and AAB (*Acetobacter tropicalis*, *A. pasteurianus* and *A. aceti*) to compose complex microbial starter cultures (Batista, Ramos, Ribeiro, Pinheiro, & Schwan, 2015; Crafack et al., 2013; Lefeber et al., 2010, 2012; Pereira, Miguel, Ramos, & Schwan, 2012; Sandhya et al., 2016; Visintin et al., 2017).

During cocoa beans fermentation, the two main soluble pulp sugars, glucose and fructose, are co-fermented to ethanol (yeast metabolism) and lactic acid (LAB metabolism), as well as other minor but important metabolites (Camu, González, et al., 2008; Nielsen et al., 2007; Schwan & Wheals, 2004). Although fructose is consumed concomitantly with glucose, the latter is depleted first, which gives rise to a discrepancy between the amount of glucose and fructose consumed during fermentation (Ardhana & Fleet, 2003; Camu, De Winter, et al., 2008; Kresnowati et al., 2013; Lagunes-Gálvez et al., 2007; Lima et al., 2011; Nielsen et al., 2007; Papalexandratou, Vrancken, de Bruyne, Vandamme, & De Vuyst, 2011; Pereira et al., 2012). Fructose may, thus, be one of the causes of long periods of cocoa beans fermentation, lasting up to 7 days (Lima et al., 2011). Other factors include the spontaneous nature of the process and the complete metabolism cycle of the three major microbial groups: sugar-to-lactic acid (LAB), sugar-to-ethanol (yeast), and ethanol-to-acetic acid (AAB) (De Vuyst & Weckx, 2016; Lima et al., 2011; Nielsen et al., 2007; Schwan & Wheals, 2004). In addition, the residual fructose can be metabolized by undesirable fungi and spoilage bacteria that proliferate when the cocoa bean fermentation actually comes to a finish (Moens, Lefeber, & De Vuyst, 2014). This overfermentation process favors unwanted production of microbial compounds, especially C3–C5 free fatty acids and extracellular proteases and lipases that might have the potential to access and degrade bean proteins and lipids. The late growth of toxigenic fungi also becomes a significant public health risk due to the production of mycotoxins, especially ochratoxin A and aflatoxin (Schwan, Pereira, & Fleet, 2015). Whether a causal relation between cocoa fermentation and fructose metabolization exists, it remains unclear.

Although LAB usually have a glucophilic metabolism, recently, a new group called fructophilic lactic acid bacteria (FLAB) has been described by Endo and Okada (2008). It includes all species of the genus *Fructobacillus* and some *Lactobacillus* (*L. kunkeei, L. brevis, L. apinorum, L. florum* and *L. plantarum*) for presenting limited or delayed growth on glucose when compared to fructose (Endo & Okada, 2008; Endo et al., 2012; Gustaw, Michalak, Polak-Berecka, & Waśko, 2018; Maeno, Dicks, Nakagawa, & Endo, 2017; Neveling, Endo, & Dicks, 2012). These microorganisms possess an incomplete gene encoding a bifunctional alcohol/acetaldehyde dehydrogenase, requiring additional electron acceptors (oxygen, fructose or pyruvate) to metabolize glucose (Endo et al., 2018). FLAB are found in fructose-rich niches, such as fruits, flowers, fermented foods and in the gastrointestinal tracts of animals consuming fructose Endo, Futagawa-Endo, & Dicks, 2009; Endo et al., 2018; Endo & Salminen, 2013). Papalexandratou, Camu, Falony, and De Vuyst (2011) first reported the presence of *Fructobacillus* sp. by culture-independent approach (PCR-DGGE) during the initial phase of cocoa beans fermentation. Then, *Fructobacillus tropaeoli* was found in Ecuadorian cocoa fermentation (Papalexandratou, Falony, et al., 2011) and *F. pseudofilculneus* occurring occasionally in cocoa fermentation in vessels (Lefeber, Gobert, et al., 2011). However, the role of FLAB during cocoa fermentation is not understood. Although, *L. plantarum* is a dominant LAB in cocoa fermentation, has not been here characterized for its fructophilic metabolism in this environment.

The aims of this work were to: (i) study the presence and diversity of FLAB in laboratory cocoa beans fermentation, (ii) isolate and characterize FLAB from fermenting material, and (iii) select and evaluate FLAB potential as cocoa starter cultures, with the aim of improving fructose consumption during fermentation process.

2. Materials and methods

2.1. Laboratory-scale cocoa fermentation and sampling

A 6-day, laboratory-scale, spontaneous cocoa bean fermentation was performed, in triplicate, based on previous works (Pereira et al., 2012; Romanens et al., 2018). Mature cocoa pods (*Theobroma cacao* L. var. Forastero) were harvested from a cacao farm located in the municipality of Ilhéus (S14°48′17″ W39°08′20″), Bahia State, Brazil, and transported within 1 to 3 days to the Bioprocess Engineering and Biotechnology Laboratory, Federal University of Paraná, Curitiba, Brazil. The pods were manually opened, and 1.8 kg of the cocoa pulp-bean transferred into a 25 cm × 16 cm × 7 cm polypropylene fermentation box containing holes in the bottom to allow drainage of the sweating. The fermentation box was maintained in a laboratory incubator Quimis 0316 M2 (Diadema, São Paulo, Brazil) for 6 days, and the temperature was adjusted every 12 h simulating on-farm process: 28 °C at 0 h, 30 °C at 12 h, 32 °C at 24 h, 35 °C at 36 h, 38 °C at 48 h, 42 °C at 60 h, 46 °C at 72 h, and 48 °C at 84 h, 96 h, 108 h and 120 h (Pereira et al., 2012). In the first 48 h, the fermentation box was kept capped with a lid to create microaerophilic conditions and favor the growth of yeasts and LAB. After this period, the lid was removed and the fermenting material was revolved every 24 h by manual kneading to promote the development of AAB and allow efficient removal of the pulp.

Ten grams of cocoa beans with adhered pulp were randomly collected every 24 h (0, 24, 48, 72, 96 and 120 h) to perform culture-dependent microbiological analyses immediately after sampling and were posteriorly stored in a freezer at −20 °C for carrying out culture-independent approaches.

2.2. Study on the presence of fructophilic lactic acid bacteria (FLAB) by culture-independent approach

Fermenting cocoa pulp bean mass samples from 0, 48 and 72 h were used to assess the presence of FLAB during laboratory-scale cocoa fermentation using Illumina high-throughput sequencing. The pulp fraction was recovered by decanting after mixing 5 mL of sterile 0.1% (w/v) peptone water with 5 g of the cocoa beans and adhering pulp. One milliliter of cocoa pulp fraction was centrifuged at 12,000*g* for 1 min (Eppendorf, Hamburg, Germany). Cell pellet was resuspended in 500 μL of Tris-EDTA, and extraction of total DNA was performed according to Carvalho Neto, Pereira, Carvalho, Soccol, and Soccol (2018). The total DNA obtained was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). A total of 20 ng of DNA was used as a template for the amplification of the V4 region of the 16S rRNA gene, using the primers 515F and 806R (Caporaso et al., 2012) with KlenTaq Master Mix (Sigma-Aldrich, Saint

Louis, MO, USA). The PCR products were quantified using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) and sequenced using the v.2 (500 cycles) Sequencing Kit (Illumina, San Diego, CA, USA) on an Illumina MiSeq (Illumina, San Diego, CA, USA) platform. After sequencing, chimeric sequences detection, removal of noises from pre-cluster and taxonomic assignment were done using standard parameters with the QIIME (Quantitative Insights Into Microbial Ecology) software package, version 1.9.0 (http://qiime.org/). Sequences with a similarity above 97% were assigned to the same operational taxonomic units (OTUs) using the SILVA database (https://www.arb-silva.de/) (Quast et al., 2013).

2.3. Isolation of presumptive FLAB

Two approaches, adapted from Endo et al. (2009), were used for the isolation of presumptive FLAB from cocoa beans fermentation. Forty grams of cocoa beans and adhering pulp from samples at 0, 24, 48, 72, 96 and 120 h were deposited into Erlenmeyer flasks containing 100 mL of FYP broth (10 g/L D-fructose, 10 g/L yeast extract, 5 g/L peptone, 2 g/L sodium acetate, 0.5 g/L Tween 80, 0.2 g/L $MgSO4{\cdot}{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}4H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.01 g/L NaCl, 0.05 g/L sodium azide, and 0.2% [v/v] nystatin [pH 6.8]) and incubated at 30 °C and 120 rpm for 24 h. After this incubation, aliquots of 1 mL were homogenized with 9 mL of 0.1% (w/v) peptone water (10^{-1} solution) and diluted serially. Then, 100 µL of each dilution were inoculated on the surface of FYP agar medium supplemented with 0.5% (w/v) $CaCO_3$ to acid production indication. Plates were incubated at 30 °C for 24–48 h, and the number of CFU was recorded, following morphological characterization and counts of each colony type obtained. The colonies that showed a clearance zone surrounding, indicating hydrolysis of $CaCO_3$, were purified on FYP agar, and stored at −80 °C in FYP broth containing 10% (v/v) glycerol.

The second approach consisted of an initial selective pressure, where each fermentation sample (0, 24, 48, 72, 96 and 120 h) were cultivated in FYP broth containing a high concentration of fructose (300 g/L D-fructose). The conditions of plating, isolation and purification of presumptive FLAB were performed as previously described for FYP broth without fructose supplementation.

2.4. Screening of carbohydrates consumption

The presumptive FLAB were selected for their efficiency in consuming glucose and fructose. Initially, the isolates were cultured on FYP agar at 30 °C for 24 h. Inoculum were prepared with the bacterial cells suspended in 0.1% (w/v) peptone water and adjusted according to the McFarland 2.0 scale (6.0×10^8 cells/mL). One milliliter of this suspension was inoculated in 9 mL adapted API 50 CHL medium (10 g/L D-glucose, 10 g/L peptone, 5 g/L yeast extract, 5 g/L sodium acetate, 2 g/L dipotassium phosphate, 2 g/L diammonium citrate, 0.2 g/L magnesium sulfate, 0.17 g/L bromocresol purple, 0.05 g/L manganese sulfate and 1 mL/L Tween 80; pH 6.8), in triplicate, and incubated at 30 °C for 96 h. The glucose-fermenting microorganisms were evaluated by the positive reaction of the color change from medium to yellow and gas production observed with Durham tubes.

Different profiles of isolates (fast or slow glucose fermentation and homofermentative or heterofermentative) were screened by fermentative capacity in medium containing fructose as the sole carbon source. Inoculum was prepared with the bacterial cells suspended in 0.1% (w/v) peptone water and adjusted according to the McFarland 0.5 scale (1.5×10^8 cells/mL). Then, one milliliter of this suspension was inoculated in 9 mL FYP broth, in triplicate, and incubated at 30 °C for 36 h. Differences in growth among the strains were monitored by recording optical density readings at 600 nm. Fructose consumption was evaluated by the colorimetric method for the determination of reducing sugars using 3,5-dinitrosalicylic acid (DNS) described by Miller (1959). After 36 h of fermentation process, the fermented obtained was stored at −20 °C for further analysis of aroma production by gas chromatography (item 2.8).

2.5. Identification of presumptive FLAB

Eighty efficient fructose-consumers isolates were selected for molecular identification by sequence analysis of the partial 16S rRNA gene. Genomic DNA was extracted from the presumptive FLAB cultured in 10 mL FYP broth at 30 °C for 24 h using phenol/chloroform method adapted by Cheng and Jiang (2006) where cell lysis occurs directly by phenol. The primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGCTACCTTGTTACGACTT-3′) were used to amplify the 16S rRNA gene region (Lane et al., 1985) in a Veriti thermal cycler (Applied Biosystems, Paisley, UK). Amplifications were performed in a final volume of 25 µL containing 5 µL of 5x GoTaq® reaction buffer supplied with $MgCl_2$ (7.5 mM) (Promega, Madison, WI, USA), 0.55 µL of dNTP Mix (10 mM) (Invitrogen, Carlsbad, CA, USA), 0.5 µL of 27F and 1492R primers (10 mM), and 0.2 µL of GoTaq® DNA Polymerase (5U/µL) (Promega). Amplicons were generated by PCR under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 93 °C for 60 s, annealing at 50 °C for 60 s, extension at 72 °C for 90 s, and final extension at 72 °C for 5 min. The PCR products were sequenced by automated capillary electrophoresis on ABI 3730xl DNA analyzer (Applied Biosystems, Paisley, UK). The sequences obtained were aligned using the BioEdit 7.7 sequence alignment editor and compared to the GenBank database. The searches were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained using the BLAST algorithm (National Center for Biotechnology Information, MA, USA).

A phylogenetic tree was constructed based on 16S rRNA gene sequences of 28 reference isolates compared to 16S rRNA sequences retrieved from NCBI database. Multiple alignments were performed using the online version of MAFFT program version 7 with the option Auto (FFT-NS-1, FFT-NS-2, FFT-NS-I, or L-INS-i). A neighbor-joining phylogenetic tree was constructed using the MEGA X version 10.1 program (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) based on the MSA file obtained in MAFFT. The evolutionary distances were computed by Maximum Composite Likelihood Method (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 2011). The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

2.6. Fructophilic properties of the isolates

The 80 isolates identified by 16S rRNA gene sequencing, encompassing *Pediococcus acidilactici* (n = 52), *P. pentosaceus* (n = 10), *L. plantarum* (n = 10), *Bacillus subtilis* (n = 4), and *Leuconostoc pseudomesenteroides* (n = 4), were inoculated in different broths to investigate their fructophilic properties using the method of Gustaw et al. (2018). After 24 h in aerobic incubation at 30 °C, bacterial cultures were centrifuged and removed from the FYP broth. The bacterial cells were resuspended in 0.1% (w/v) peptone water, and the same optical density of 0.5 was set at 600 nm. Growth characteristics of the strain were determined in three different media: FYP, GYP (containing 10 g/L of D-glucose instead of D-fructose) and GYP-P supplemented with 0.5% pyruvate. Two hundred microliters of each medium were transferred to microplates, in triplicate, and the wells were inoculated with 30 µL of the bacterial suspension. The experiment was performed under aerobic and anaerobic conditions by measuring the optical density at 600 nm every 2 h for 24 h. Anaerobic conditions were obtained by cutting off access to oxygen with a few drops of liquid vaseline.

2.7. Rep-PCR analysis and computer-assisted analysis of genomic fingerprints

Ten *L. plantarum* fructophilic strains were typed at strain level using repetitive extragenic palindromic (Rep)-PCR technique using a $(GTG)^5$ primer (5′-GTG GTG GTG GTG GTG-3′) (Versalovic, Schneider, de Bruijn, & Lupski, 1994). The amplifications were performed following the conditions proposed by Pereira et al. (2017). Amplicons were loaded and separated by electrophoresis in 1.8% (w/v) agarose gels using DNA markers GeneRuler 50 bp DNA Ladder (Invitrogen, Carlsbad, CA, USA) as a reference. Then, the PCR products were stained with ethidium bromide (10 mg/mL) and scanned using a Fluoro Image Analyzer FLA-5000 (Fuji Photo Film Co.).

The genomic fingerprints obtained were converted to a two-dimensional binary matrix (1 = presence of a band; 0 = absence of a band). Dice (Sorensen) coefficient was used to calculate the similarity matrices using the unweighted pair group method with the arithmetic averages clustering algorithm (UPGMA). Computer-assisted analysis was performed with SYSTAT® version 10.0 program for Windows.

2.8. Selection of aroma-producing isolates

The ten *L. plantarum* fructophilic strains were selected for their capacity to produce volatile aroma compounds in FYP medium. The extraction of volatile compounds was performed using a headspace (HS) vial coupled to a SPME fiber (CAR/PDMS df75 µm partially crosslinked, Supelco, Saint Louis, MO, USA). For each determination, 2 mL of sample was stored in a 20 mL HS vial, in duplicate. The SPME fiber was exposed for 30 min at 60 °C. The compounds were thermally desorbed into the gas chromatograph injection system gas phase (CGMS TQ Series 8040 and a 2010 Plus GC–MS; Shimadzu, Tokyo, Japan) at 260 °C. The column oven temperature was maintained at 60 °C during 10 min, followed by two heating ramps of 4 and 10 °C/min until reaching the temperatures of 100 and 200 °C, respectively. The compounds were separated on a column 95% PDMS/5% PHENYL (30 m × 0.25 mm × 0.25 mm film thickness). The GC was equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). The compounds were identified by comparison to the mass spectra from library databases (Nist'98 and Wiley7n).

2.9. Performance of selected FLAB in cocoa beans fermentation

The selected aroma-producing FLAB, *L. plantarum* LPBF 35, was introduced into a cocoa beans fermentation process to evaluate the efficiency of fructose consumption. A glucophilic strain, *P. acidilactici* LPBF 66, and a spontaneous cocoa fermentation were used as positive and negative controls, respectively. The frozen strains were initially reactivated from −80 °C to FYP (fructophilic strain) and MRS (glucophilic strain) broths for 48 h at 30 °C. Afterwards, the strains were transferred to 500 mL of FYP and MRS broths, respectively. After incubation, the cells were centrifuged at 7.000*g* for 10 min and washed twice with a buffer phosphate pH 7.0 to remove any culture medium residue.

Laboratory-scale cocoa beans fermentations (as previously described) were conducted with the inoculation of *L. plantarum* LPBF 35 and *P. acidilactici* LPBF 66. A spontaneous process was carried out as a negative control. The initial bacterial cell concentration was adjusted to 1.5×10^9 cells/mL (O.D. = 0.1). Fermentations were carried out in triplicate at 30 °C for 72 h. The difference in glucose and fructose consumption, and also lactic acid production between the three treatments was performed by high-performance liquid chromatograph (HPLC). Filtered samples collected from 0, 12, 24, 36, 48, 60 and 72 h were injected into HPLC system equipped with an Aminex HPX 87H column (300 × 7.8 mm; Bio-Rad, Richmond, CA, USA) and a refractive index (RI) detector (HPG1362A; Hewlett-Packard Company, Palo Alto, CA, USA). The column was eluted in an isocratic mode with a mobile phase of 5 mM H_2SO_4 at 60 °C and a flow rate of 0.6 mL/min.

2.10. Statistical analysis

The data obtained by the target metabolites analysis were made by post-hoc comparison of means by the Duncan's test. The inoculation efficiency of *L. plantarum* LPBF 35 and *P. acidilactici* LPBF 66 after 72 h of fermentation was analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's test. Statistical analyses were done using STATISTICA 7 StatSoft software (STATSOFT, 2007). The level of significance was established using a p-value < 0.05.

3. Results and discussion

3.1. Characteristics of sample sequencing data and LAB community structure

A total of 55,703 sequence reads obtained by Illumina-based amplicon sequencing were clustered in 316 OTUs at 97% sequence similarity. The rarefaction analysis indicated a satisfactory coverage of all the samples, suggesting that the majority of bacterial communities was covered (Supporting Information, Fig. S1). The identified microbial OTUs, excluding the unclassified, were divided into five phyla, 45 families and 58 genera, after searching in the SILVA database. The complete list of bacteria at the genus level is shown in the supplementary material (Supporting Information, Table S1). Analysis of phyla revealed a high relative abundance of Proteobacteria (82.02%) during the whole cocoa fermentation process, followed by Firmicutes (17.62%), Actinobacteria (0.32%) and Bacteroidetes (0.02%). Proteobacteria was recently reported as the dominant phylum in Brazil and in four regions of cocoa production in Africa (Agyirifo et al., 2019; Bortolini, Patrone, Puglisi, & Morelli, 2016; Illeghems, De Vuyst, Papalexandratou, & Weckx, 2012; Serra et al., 2019). The role of Proteobacteria during the cocoa bean fermentation includes the production of degrading enzymes and oxidation of ethanol to acetic acid (Illeghems, Weckx, & De Vuyst, 2015; Serra et al., 2019).

Fructophilic lactic acid bacteria (FLAB) belong to the phylum Firmicutes, a very diverse group of bacteria with a low G + C content ($>$ 50 mol%) in their genome (Seong et al., 2018). Fig. 1 shows filtered sequences related to Firmicutes found in this study. *Pediococcus* was the dominant genera reaching 85% relative abundance within 48 h of fermentation. This is the first study to report the dominance of *Pediococcus* in cocoa fermentation, which have been found in lower prevalence in Brazil and Nigeria (Agyirifo et al., 2019; Illeghems et al., 2012, 2015; Kostinek et al., 2008; Miguel et al., 2017; Papalexandratou, Camu, et al., 2011; Papalexandratou, Falony, et al., 2011; Papalexandratou, Vrancken, et al., 2011; Passos, Silva, Lopez, Ferreira, & Guimarães, 1984; Serra et al., 2019). Other Firmicutes members that were found included *Paenibacillus*, *Lactobacillus*, *Bacillus* and *Leuconostoc*.

Fructobacillus was detected only at 0 h (1.65%) of fermentation. Other studies, which used culture-independent approaches, also found low population of *Fructobacillus*, and only at the beginning of cocoa fermentation in Brazil, Ecuador, Malaysia, Ivory Coast and Ghana (Agyirifo et al., 2019; Menezes et al., 2016; Papalexandratou, Camu, et al., 2011; Serra et al., 2019). These results indicate that, although *Fructobacillus* is present in cocoa pulp, they are not able to compete with other well-adapted glucophilic bacteria and yeasts. The high ethanol content (6.5–25 mg/g of pulp) produced by yeast may favor the growth of alcohol-tolerant LAB, such as certain species of *Lactobacillus, Leuconostoc,* and *Pediococcus* (Endo, Tanaka, Oikawa, Okada, & Dicks, 2014). In addition, FLAB can compete for fructose with other strict or facultative heterofermentative LAB species, such as *L. fermentum* and *P. acidilactici*¸ which use hexose as an alternative external electron acceptor (De Vuyst & Weckx, 2016).

Lactobacillus is another LAB group reported to have members with fructophilic characteristics, including *L. florum*, *L. brevis, L. kunkeei, L. apinorum* and *L. plantarum* (Endo et al., 2010, 2012; Gustaw et al., 2018; Maeno et al., 2017; Neveling et al., 2012). In general, *L. plantarum* and

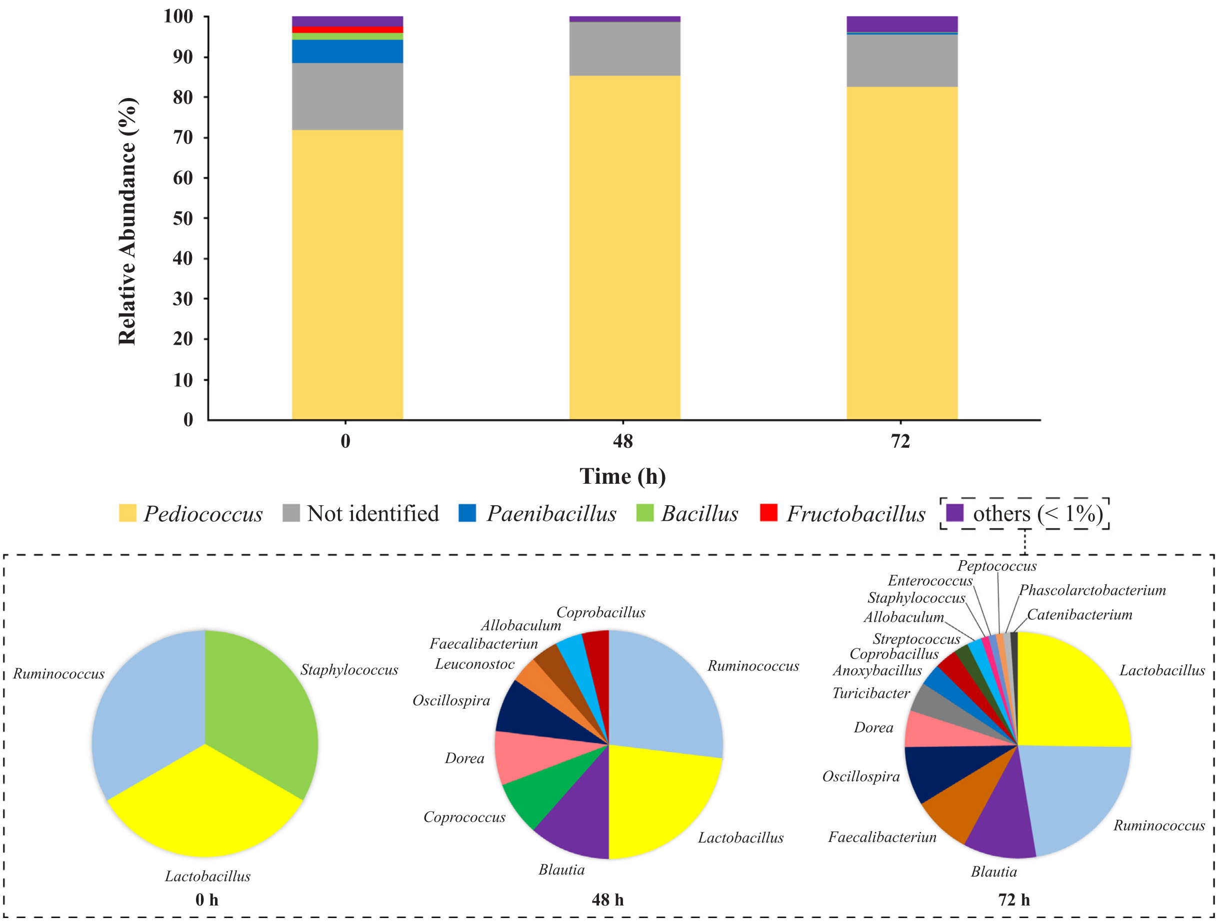

Fig. 1. Firmicutes diversity by Illumina high-throughput sequencing during laboratory-scale cocoa beans fermentation at 0, 48 and 72 h.

L. fermentum have frequently been reported as the dominant species in cocoa fermentations around the world (Bortolini et al., 2016; Camu et al., 2007; Hamdouche et al., 2015; Illeghems et al., 2015; Meersman et al., 2013; Nielsen et al., 2007; Papalexandratou, Camu, et al., 2011; Pereira et al., 2012; Pereira, Magalhães, Almeida, Coelho, & Schwan, 2013; Serra et al., 2019; Visintin et al., 2016). The lower *Lactobacillus* abundance found in this study can be associated with the reported high prevalence of *Pediococcus*. It is known that the growth of *Pediococcus* species during wine fermentation contribute to subsequent inhibition of other LAB, through production of hydrogen peroxide, bacteriocins and other antibiotic-like substances. However, toxicity has only a transitory effect, *i.e.,* it did not kill and eliminate the bacteria, it only decreases the growth rate and lowered the final population density (Lonvaud-Funel & Joyeux, 1993).

3.2. Isolation and identification of fructose-consuming LAB

The initial microbial count on FYP agar plates was 2.14 log CFU/mL and reached a maximum count of 9.0 log CFU/mL after 72 h, followed by a drop to 6.6 log CFU/mL by 120 h (Supplementary Fig. S2). Overall, 204 isolates were randomly picked up from different fermentation times and evaluated to grow in medium containing glucose as the sole carbon source. A total of 80 isolates, having low growth efficiency in glucose, were selected since FLAB feature a limited growth when glucose is present as a single carbon source (Endo et al., 2009, 2018). Subsequently, these 80 isolates were evaluated for fructose consumption efficiency (Supplementary Table S2). From an initial fructose concentration of 10 g/L, all the isolates were able to consume > 50% (< 5 g/L) after 36 h of fermentation. In general, the isolates identified as *L. plantarum* showed a more efficient consumption rate with residual fructose reaching values inferior to 4 g/L, which corroborates to the potential fructophilic characteristic attributed to this group (Endo et al., 2012, 2018).

The 80 fructose-fermenting isolates were identified by 16S rRNA gene sequencing as *P. acidilactici* (n = 52; 16S rRNA gene 99% sequence similarity), *P. pentosaceus* (n = 10; 16S rRNA gene 99% sequence similarity), *L. plantarum* (n = 10; 16S rRNA gene 99% sequence similarity), *B. subtilis* (n = 4; 16S rRNA gene 99% sequence similarity), and *Leu. pseudomesenteroides* (n = 4; 16S rRNA gene 99% sequence similarity) (Supplementary Table S2). Although the genus *Fructobacillus* was found by Illumina approach, it was not isolated on FYP medium. The FYP has been successfully applied for isolation of FLAB from flowers and bee guts (Endo et al., 2009, 2010, 2012; Filannino, Cagno, Addante, Pontonio, & Gobbetti, 2016; Neveling et al., 2012). These are sources with significantly lower loads of LAB than cocoa fermentation with generally observed populations above 10^8 CFU/g (Ardhana & Fleet, 2003; Camu et al., 2007; Lefeber et al., 2010; Papalexandratou, Camu, et al., 2011; Papalexandratou, Vrancken, et al., 2011; Pereira et al., 2012). The nutrient-rich medium of cocoa pulp may have favored the growth of faster growing LAB (*i.e.*, *Pediococcus*) on FYP agar plates at the expense of slow growing of *Fructobacillus*. In any way, with these results, it is possible to speculate that the diversity of bacterial species in cocoa beans fermentation is inevitably underestimated using standard cultivation methods and that organisms of key importance to the community and the entire ecosystem may be overlooked. The use of dilute nutrient media techniques can be tested for recovery of *Fructobacillus* and other slow growing microbial species from cocoa fermentation (Connon & Giovannonis, 2002; Rappe, Connon, Vergin, & Giovannoni, 2002; Zengler et al., 2002). These include filtration methods (Hahn, Stadler, Wu, & Pockl, 2004), density-gradient

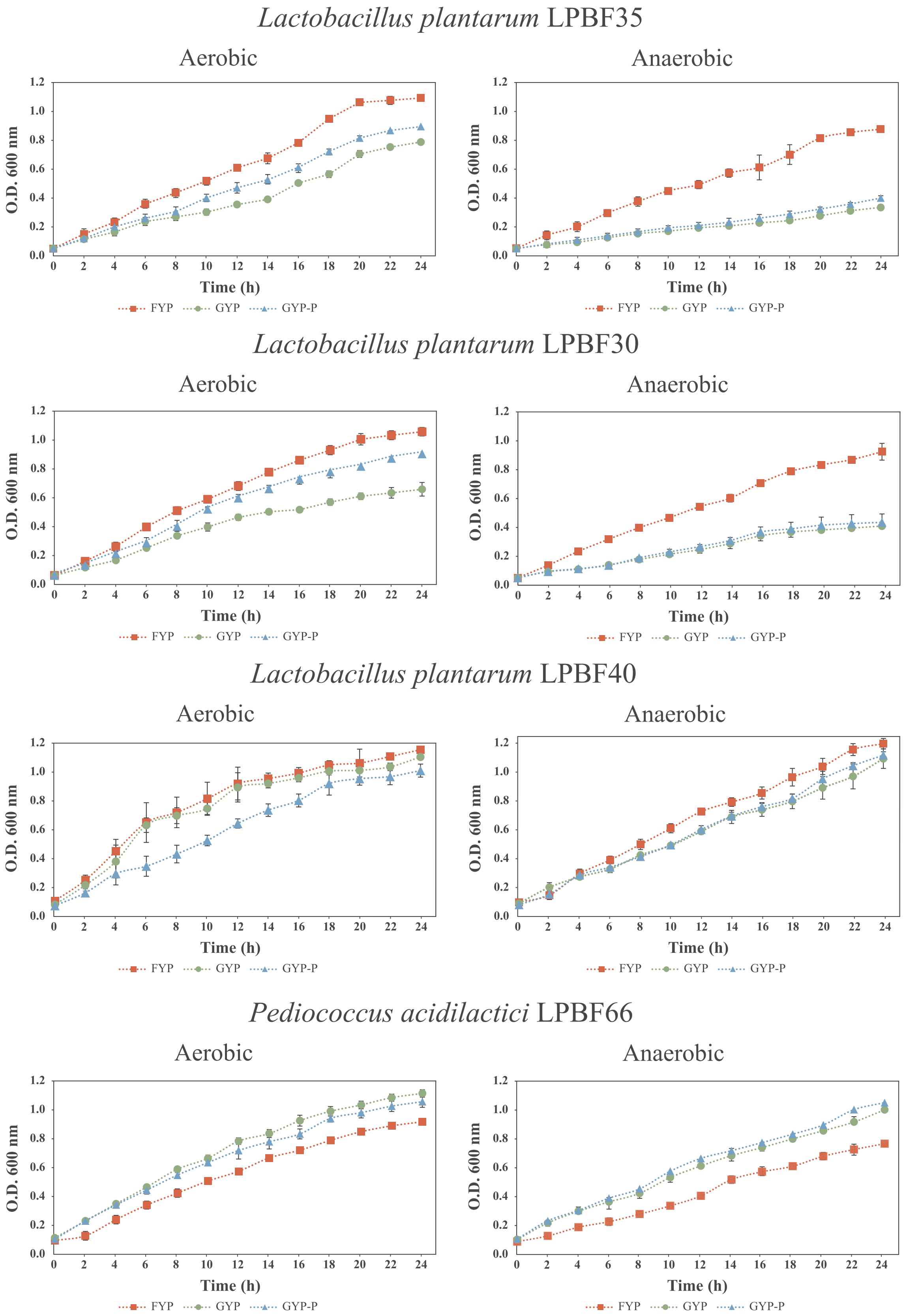

Fig. 2. Growth curves model of facultative fructophilic *L. plantarum* strains (LPBF 35, LPBF 30, and LPBF 40) and non-fructophilic *P. acidilactici* LPBF 66 under aerobic and anaerobic conditions. FYP = fructose yeast peptone; GYP = glucose yeast peptone; GYP-P = glucose yeast peptone supplemented with pyruvate.

centrifugation or elutriation and extinction-dilution, whereby samples are diluted, ideally down to single cells, before their culture in isolation (Ben-Dov, Kramarsky-Winter, & Kushmaro, 2009; Connon & Giovannonis, 2002; Song, Oh, & Cho, 2009; Wang, Hammes, Boon, Chami, & Egli, 2009).

3.3. Fructophilic properties

All 10 *L. plantarum* strains showed fructophilic proprieties (preferring D-fructose to D-glucose as a main source of growth), while *P. acidilactici*, *B. subtilis* and *Leu. pseudomesenteroides* strains were glucophilic (Fig. 2). Among the 10 strains, two of them (*L. plantarum* LPBF 30 and LPBF 35) grew faster in FYP than in both GYP and GYP-P, under anaerobic and aerobic conditions. The other eight *L. plantarum* strains showed similar growth in FYP and GYP under aerobic condition, but faster in FYP under anaerobic condition. The characteristics of all the 10 *L. plantarum* strains classified them as “facultatively” fructophilic bacteria, which means they can grow on glucose without an external electron acceptor but the growth on fructose is faster (Gustaw et al., 2018). Obligate FLAB group, which includes all species of *Fructobacillus* and *L. kunkeei*, require external electron acceptors (*i.e.,* oxygen, fructose, pyruvate, p-coumaric and caffeic acid) in order to maintain the balance of the $NAD^+/NADH$ ratio associated with the loss of the alcohol/acetaldehyde dehydrogenase gene (Filannino, Cagno, Tlais, Cantatore, & Gobbetti, 2019; Maeno et al., 2016). The main end-metabolites produced by obligate FLAB are acetate and CO_2, while ethanol is hardly produced (Endo et al., 2012, 2018; Maeno et al., 2016). On the other hand, facultative fructophilic properties are not associated with the loss of the alcohol/acetaldehyde dehydrogenase gene, and species of this group, including some strains of *L. florum, L. plantar*um and *L. brevis*, can grow on glucose without an external electron acceptor. The end-metabolites of facultative FLAB from glucose includes high rates of lactic acid, ethanol, acetic acid and CO_2 (Endo et al., 2014, 2018; Filannino et al., 2019; Neveling et al., 2012).

3.4. Genotypic characterization of presumptive FLAB

Taxonomic strain characterization was performed by comparing the sequences of each isolate with those reported in the NCBI Reference. Three distinct clusters were formed according to neighbor-joining method, namely G1 - *Lactobacillus* group, G2 - *Pediococcus* group, and G3 - *Leuconostoc* group (Fig. 3). Interestingly, the two strains that showed fast growth in FYP under anaerobic and aerobic conditions, *L. plantarum* LPBF 30 and LPBF 35, were grouped into a distinct subcluster among the G1 group, along with *L. plantarum* LPBF 42. This demonstrates a genetic variation of FLAB within the 16S rRNA gene, which can be used to show the strains differences. $(GTG)_5$-rep-PCR genomic fingerprinting was used to confirm the differentiation of fructophilic strains (Fig. 4). A computer-assisted analysis clearly differentiated *L. plantarum* LPBF 30 and LPBF 35 from the other strains. In the same way, *L. plantarum* LPBF 42 was also grouped close to both *L. plantarum* LPBF 30 and LPBF 35, corroborating the observed data from the 16S rRNA gene phylogenetic tree. In addition, both LPBF 35 and LPBF 30 presented up to seven fewer fragments than the glucophilic strains. FLAB have significantly fewer genes for carbohydrate metabolism than other LAB, especially due to the lack of complete phosphotransferase system (PTS) transporters (Endo et al., 2015, 2018; Filannino et al., 2019; Maeno et al., 2016, 2017). Thus, the ongoing reduction of the genome called “reductive evolution”, together with acquisition or overexpression of genes (Gustaw et al., 2018; Van de Guchte et al., 2006), may explain the differentiation of both strains. (GTG)5-rep-PCR genomic fingerprinting revealed itself as a promising genotypic tool for evaluating the rapid and reliable speciation of FLAB.

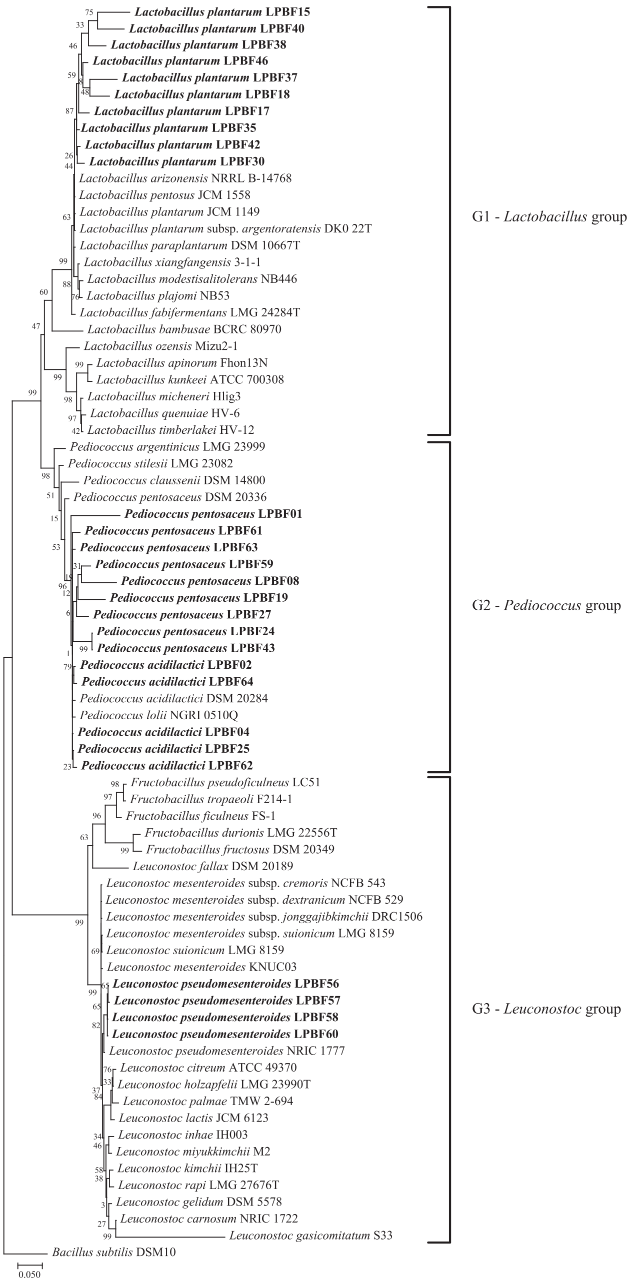

Fig. 3. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships of LAB isolated from laboratory-scale cocoa beans fermentation. Bootstrap values (%) based on 1000 replications are shown at branch points. The substitution model used was Kimura 2-parameter model. Bar = 0.05% sequence divergence.

3.5. Volatile compounds production and coffee beans fermentation conducted with selected FLAB

LAB are known for producing a wide range of volatile aroma compounds during cocoa beans fermentation, which contribute for the formation of desirable sensory notes in the composition of chocolate. Aroma production has been used as an important criterion for selecting LAB to be used in cocoa fermentation (Afoakwa, Paterson, Fowler, & Ryan, 2008; De Vuyst, Lefeber, Papalexandratou, & Camu, 2010; Janek, Niewienda, Wöstemeyer, & Voigt, 2016; Lagunes-Gálvez et al., 2007; Lefeber, Gobert, et al., 2011). The metabolism of aroma formation for all ten fructophilic strains was characterized and reported in Table 1. SPME-GC/MS analysis enabled the identification of a total of 21

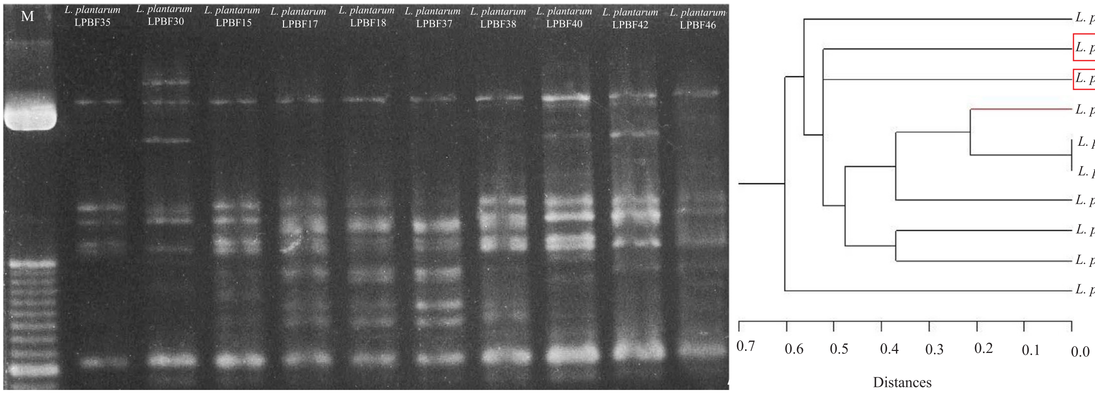

Fig. 4. (GTG)5-rep-PCR electrophoresis band pattern (A) and clustering (B) according with the UPGMA analysis based on Dice coefficient of strains of *L. plantarum* isolated from cocoa beans fermentation. Red lines indicate fructophilic *L. plantarum strains* LPBF 30 and LPBF 35. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compounds, including organic acids (5 compounds), alcohols (5 compounds), aldehydes (4 compounds), furans (1 compound), esters (4 compounds) and ketones (3 compounds). Some flavor-active compounds (*i.e.,* acetaldehyde, ethyl acetate, nonanal, and octanoic acid) are reported in literature for attributing desirable fruity and sweetish sensory notes, thus enriching and modulating the flavor of chocolate (Camu, De Winter, et al., 2008; Menezes et al., 2016; Rodriguez-Campos, Escalona-Buendía, Orozco-Avila, Lugo-Cervantes, & Jaramillo-Flores, 2011). Maeno et al. (2016) showed that fructophilic species of *L. kunkeei* possess high number of genes related to amino acids transport and catabolism, which could also explain the elevated production of aldehydes and carboxylic acids. Those low molecular weight compounds are strictly correlated with the catabolism of amino acids in LAB (Pereira et al., 2019).

Among the two faster fructose-consuming strains (LPBF 30 and LPBF 35), *L. plantarum* LPBF 35 was introduced as a starter culture in laboratory cocoa beans fermentation due to its higher production ($p > 0.05$) and diversity of volatile aroma compounds. The metabolism of sugar consumption and lactic acid production was compared to fermentations conducted with a glucophilic bacterium (*P. acidilactici* LPBF 66) and a spontaneous process (Fig. 5). The observed increase in the concentration of glucose (from approx. 103.19 mg/g up to 107.96 mg/g) and fructose (from approx.101.64 mg/g up to 105.31 mg/g) at 12 h of fermentation can be attributed to the hydrolysis of sucrose, pectin and other complex polysaccharides present in cocoa pulp (Pereira et al., 2012; Pereira, Magalhães et al., 2013; Rodriguez-Campos et al., 2011). After 24 h, fructose was more rapidly metabolized ($p > 0.05$) in fermentation containing the fructophilic strain in comparison with the glucophilic strain and the spontaneous process; the opposite was observed for glucose metabolism. Therefore, residual fructose was not observed in the FLAB process. Previous studies have reported a residual fructose content (5 mg/g up to 17 mg/g) at 120 h of spontaneous cocoa fermentation (Ardhana & Fleet, 2003; Camu, De Winter, et al., 2008; Kresnowati et al., 2013; Lagunes-Gálvez et al., 2007; Lima et al., 2011; Nielsen et al., 2007; Papalexandratou, Camu, et al., 2011; Pereira et al., 2012). Thus, the use of FLAB can assist in the fructose metabolism, contributing to the drying of cocoa beans. In addition, residual fructose can be metabolized by undesirable fungi and spoilage bacteria that proliferate in the end of the fermentation process.

Metabolism of organic acids is another mechanism by which LAB are considered to impact on cocoa bean composition and quality. The utilization of citric acid and production of lactic acid are considered the two main functions of LAB, decreasing the acidity of the pulp, and equilibration of the bean pH to values around 5.0–5.5, which are considered optimal for endogenous proteolytic and other enzymatic activities (De Vuyst et al., 2010; Schwan et al., 2015). Although the different fermentative processes showed similar profile of organic acid metabolism, citric acid was most efficiently metabolized in the process by *P. acidilactici* LPBF 66 (residual concentration of 16.40 mg/g, 9.73 mg/g and 13.91 mg/g in *L. plantarum* LPBF 35, *P. acidilactici* LPBF 66 and spontaneous process, respectively). On the other hand, both fructophilic and glucophilic inoculated processes achieved similar lactic acid concentration (approx. 50 mg/g at 36 h), which was significantly higher than the spontaneous process (37.71 mg/g). These results demonstrate that FLAB has an ideal profile of organic acid metabolism for cocoa fermentation.

4. Conclusions

FLAB of the genera *Fructobacillus* and *Lactobacillus* inhabit cocoa pulp; however, *Fructobacillus* remains as a yet-to-be-cultivated genus from this environment. The bioprospecting of fructophilic *L. plantarum* promoted a faster utilization of fructose and ideal profile of organic acid metabolism associated with cocoa fermentation. The exploration of these new taxa will promote the best opportunities to isolate novel microorganisms with functional proprieties and, ultimately, their use as improved starters.

CRediT authorship contribution statement

Jéssica A. Viesser: Conceptualization, Writing - original draft. **Gilberto V. de Melo Pereira:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition. **Dão Pedro de Carvalho Neto:** Conceptualization, Writing - original draft. **Luciana P. de S. Vandenberghe:** Writing - review & editing, Funding acquisition. **Vasco Azevedo:** Conceptualization, Writing - review & editing. **Bertram Brenig:** Conceptualization, Writing - review & editing. **Hervé Rogez:** Conceptualization, Writing - review & editing. **Aristóteles Góes-Neto:** Conceptualization, Writing - review & editing. **Carlos Ricardo Soccol:** Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1

Percentage (%) of the area of volatile compounds produced by *L. plantarum* strains, isolated from laboratory-scale cocoa beans fermentation, in FYP broth medium.

	Control	*L. plantarum* LPBF 35	*L. plantarum* LPBF 30	*L. plantarum* LPBF 15	*L. plantarumv* LPBF 17	*L. plantarum* LPBF 18	*L. plantarum* LPBF 37	*L. plantarum* LPBF 38	*L. plantarum* LPBF 40	*L. plantarum* LPBF 42	*L. plantarum* LPBF 46
Volatile Organic Compound											
Acids											
Octanoic acid	9.5 ± 2.3a	7.6 ± 4.1ab	ND	ND	ND	ND	ND	3.1 ± 0.5a	ND	4.1 ± 0.7a	ND
Oxalic acid derivative	12.2 ± 5.4a	3.4 ± 0.5ab	5.4 ± 1.6ab	ND	3.7 ± 0.2ab	ND	ND	2.7 ± 0.2b	0.9 ± 0.01c	ND	3.7 ± 0.8b
2-Methyl butanoic acid	5.0 ± 3.6a	ND	6.0 ± 1.8ab	ND	6.0 ± 0.9b	14.9 ± 5.9c	12.1 ± 0.7c	7.4 ± 1.3b	8.9 ± 1.9c	8.7 ± 2.6bc	5.9 ± 1.0b
3-Methyl butanoic acid	10.8 ± 4.1a	10.6 ± 3.9b	5.8 ± 2.2a	4.4 ± 2.7a	8.0 ± 0.4b	13.1 ± 2.9b	12.1 ± 1.6b	7.6 ± 0.8ab	7.3 ± 1.7ab	10.0 ± 4.2ab	5.3 ± 0.4a
2-Decenoic acid	5.9 ± 2.3a	ND	ND	3.7 ± 1.4a	ND	ND	ND	4.0 ± 0.3a	ND	ND	ND
Aldehydes											
Acetaldehyde	7.2 ± 0.9a	7.3 ± 3.0b	7.8 ± 3.1b	6.0 ± 1.6b	6.9 ± 1.5b	7.5 ± 1.9b	6.7 ± 1.2b	5.6 ± 3.0ab	4.8 ± 1.1ab	8.5 ± 1.5b	6.7 ± 3.5ab
Benzaldehyde	4.0 ± 1.4a	9.7 ± 1.9b	10.5 ± 2.9bc	7.4 ± 2.8bc	6.5 ± 0.7bc	8.3 ± 3.7bc	8.1 ± 0.5bc	10.5 ± 2.8bc	10.0 ± 3.0b	10.0 ± 2.6b	9.2 ± 3.1bc
Nonanal	6.7 ± 4.1a	8.3 ± 1.2b	10.3 ± 0.7b	8.5 ± 0.2b	9.3 ± 3.2bc	13.9 ± 5.1bc	10.5 ± 1.2b	8.3 ± 3.3b	8.7 ± 2.0b	ND	8.8 ± 1.4b
Decanal	10.8 ± 2.7ab	7.1 ± 2.7bc	9.8 ± 0.4c	8.0 ± 1.8bc	ND	10.4 ± 2.1ab	8.4 ± 0.9ab	ND	8.0 ± 2.8c	6.3 ± 0.4ab	4.5 ± 1.6ab
Alcohols											
1-Hexanol	5.4 ± 2.3a	5.1 ± 2.3ab	5.6 ± 3.4ac	8.0 ± 0.4c	6.0 ± 1.7bc	ND	ND	3.3 ± 0.2a	ND	0.4 ± 0.01a	7.8 ± 0.4bc
1-Heptanol	3.6 ± 0.9a	5.1 ± 1.4bc	8.7 ± 3.8bc	11.0 ± 0.7c	10.8 ± 2.2c	ND	15.6 ± 5.1c	5.8 ± 0.5c	3.7 ± 0.4b	3.0 ± 0.2b	5.5 ± 1.0b
1-Octanol	4.5 ± 1.8a	6.0 ± 3.4b	ND	10.6 ± 1.4c	11.4 ± 0.9c	9.6 ± 1.3b	ND	6.9 ± 1.0b	2.0 ± 0.1a	5.4 ± 1.3b	9.6 ± 2.7bc
1-Decanol	6.3 ± 2.7a	5.3 ± 2.8b	ND	7.4 ± 2.3b	ND	ND	ND	1.5 ± 0.1a	5.9 ± 1.3b	2.6 ± 0.9a	ND
2-Propyl-1-pentanol	2.3 ± 0.2a	ND	8.3 ± 2.9b	ND	ND	ND	ND	2.9 ± 0.1a	3.5 ± 0.7b	ND	ND
Esters											
Ethyl acetate	ND	6.4 ± 1.2b	ND	5.0 ± 1.1ab	3.2 ± 1.1a	6.7 ± 1.1ab	8.6 ± 1.4ab	6.5 ± 1.3ab	ND	6.3 ± 0.7b	3.1 ± 1.4a
Methyl acetate	ND	3.0 ± 0.1a	4.0 ± 1.1a	4.2 ± 1.9a	4.7 ± 1.9a	7.2 ± 0.5a	5.3 ± 2.6a	7.6 ± 1.5b	4.1 ± 0.9a	7.9 ± 0.2b	ND
3-Methyl-1-butanol acetate	ND	ND	ND	ND	6.3 ± 0.9b	ND	7.2 ± 1.6b	ND	5.9 ± 2.4ab	5.2 ± 2.2ab	2.2 ± 1.0a
Furans											
2-Pentylfuran	5.9 ± 2.7a	4.4 ± 2.5ab	ND	ND	ND	ND	ND	5.4 ± 1.8ab	5.0 ± 1.5ab	5.9 ± 1.1b	ND
Ketones											
2-Heptanone	ND	ND	8.3 ± 0.7b	6.7 ± 1.6bc	7.6 ± 2.6bc	5.6 ± 1.3a	ND	7.6 ± 1.5bc	8.3 ± 3.5bc	5.0 ± 0.9a	9.6 ± 4.3c
2-Octanone	ND	6.5 ± 2.3bc	ND	ND	ND	ND	1.9 ± 0.1a	3.1 ± 0.1a	6.3 ± 1.1b	6.3 ± 1.3b	10.8 ± 3.7c
2-Nonanone	ND	4.1 ± 0.5b	9.5 ± 1.1b	9.1 ± 3.2bc	9.5 ± 3.9bc	2.9 ± 0.8a	3.5 ± 0.1a	ND	6.7 ± 1.5b	4.6 ± 0.2b	7.3 ± 2.2b

*ND = not detected. Means of duplicate in each row bearing the same letters are not significantly different (p > 0.05) from one another using Duncan's Test (mean ± standard variation).

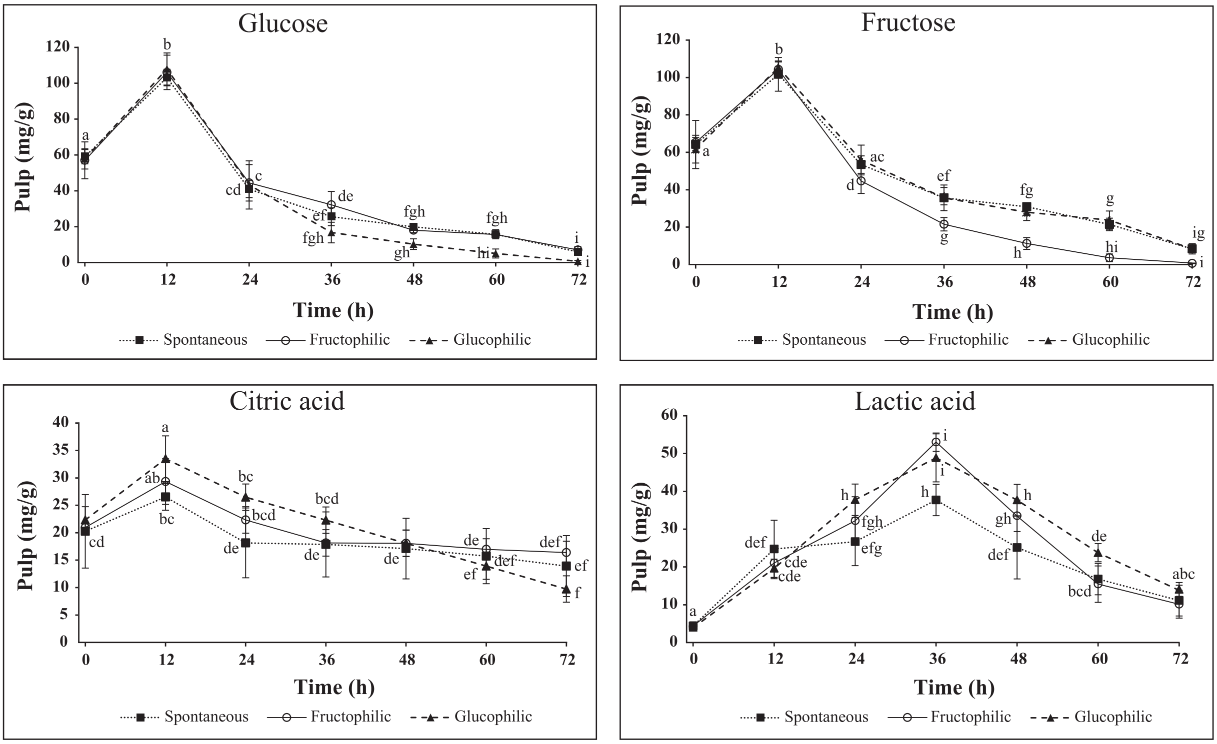

Fig. 5. Profile of consumption of glucose and fructose, and metabolism of citric acid and lactic acid, during cocoa beans fermentation conducted with *L. plantarum* LPBF 35 (fructophilic bacterium), *P. acidilactici* LPBF 66 (non-fructophilic bacterium) and spontaneous process. Means of triplicate in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Duncan's Test.

for the research scholarship.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109478.

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